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PHYSIOLOGICAL AND BIOCHEMICAL INVESTIGATIONS OF THE EGG JELLY RELEASE IN *PENAEUS AZTECUS*

JOHN W. LYNN AND WALLIS H. CLARK JR.

Department of Zoology and Physiology, Louisiana State University, Baton Rouge, Louisiana 70803 and Bodega Marine Laboratory, University of California, Bodega Bay, California 94923

ABSTRACT

Following contact with seawater, *Penaeus aztecus* ova undergo a massive release of extracortical jelly precursor material which is transformed into a layer of jelly-like material surrounding the ova. Release and dissipation of the precursors can be irreversibly inhibited by the protease inhibitors N-a-p-tosyl-L-lysine chloromethyl ketone and soybean trypsin inhibitor, implicating trypsin-like proteases in the process. Treatment with the less-specific enzyme inhibitor phenylmethyl sulfonyl fluoride also irreversibly inhibits the release of the cortical material. Jelly precursor in whole mature ovaries stain positive with PAS. Staining with alcian blue reveals acid mucopolysaccharides in the investment coat of the ova but not in the jelly precursors. Precursors isolated from whole mature ovaries are approximately 25–30% carbohydrate (anthrone sulfuric acid reaction) and 70–75% protein (Lowry's and Bradford's protein determinations). No sialic acids are detected in the isolates (thiobarbituric acid assay). Trypsin is effective in dissipating the precursor isolates. Amino acid analysis reveals high ratios of cysteic acid. Significant biochemical differences between *P. aztecus* egg jelly material and sea urchin egg jelly are discussed.

INTRODUCTION

Spawning of eggs from the ovary of the penaeid shrimp into the surrounding seawater results in a dramatic and massive release of a jelly precursor from extracellular cortical crypts (Hudinaga, 1942; Clark *et al.*, 1980, 1984). This release is initiated by contact with seawater. According to the morphological descriptions of Clark *et al.* (1980, 1984) a membrane fusion event is not involved. Initially, the jelly precursor components are stored in crypts in the surface of the mature egg separated from the environment by only a thin egg investment coat (Clark *et al.*, 1980, 1984). Transformation of the jelly precursors into the jelly coat surrounding the eggs of *P. setiferus* and *P. aztecus* has been demonstrated to be a Mg^{+2}-dependent event (Clark and Lynn, 1977). The rod-shaped jelly precursors contained in ovarian eggs of *P. setiferous* were originally described by King (1948) as peripheral bodies and recognized as jelly precursors by Hudinaga (1942) and later investigators. The biochemical composition and physiology of the release of the precursors is still poorly understood. We now report new data on the physiological parameters involved in the egg jelly release and present biochemical data on the composition of the released material.

MATERIALS AND METHODS

Animal collection

Using a standard otter trawl, brown shrimp (*P. aztecus*) were collected 80–100 miles south of Galveston, Texas. Animals were transported to the laboratory in a 150-

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Abbreviations: periodic acid-Schiff, PAS; isolation medium, IM; ethylene-diaminetetraacetic acid, EDTA; soybean trypsin inhibitor, SBTI; N-α-p-tosyl-L-lysine chloromethyl ketone, TLCK; phenylmethyl sulfonyl fluoride, PMSF.

gal tank at 13–20°C. In the laboratory, gravid female shrimp were placed in aerated, inverted 5-gal carboys, and the water temperature was slowly raised to 28°C to induce spawning. Ova obtained from these animals were used for investigations on jelly precursor release.

Jelly precursor isolation

Mature ovaries suspended in an isolation medium (IM) (500 m*M* NaCl, 9 m*M* $CaCl_2$, 14 m*M* KCl, 15 m*M* $MgCl_2$, and 10 m*M* Tris, pH 7.6) containing 30% sucrose or 35% glycerol were homogenized with a Potter-Elvehjem tissue grinder. The homogenate was centrifuged (1000 × *g* for 5 min). The pellet was resuspended in IM, layered over IM containing 60% sucrose or 70% glycerol, and centrifuged (8000 × *g* for 60 min). The resulting pellet, consisting mainly of the jelly precursor, was washed four times to remove contaminant sucrose or glycerol. These isolates were either used immediately or freeze-dried and stored at −80°C. All isolates were assayed for purity using light and electron microscopy.

Protein analysis

A Lowry's total protein reaction (Lowry *et al.*, 1951) was performed on the isolated precursor (40 mg/ml) and spectrophotometrically measured on a Coleman 124 double beam spectrophotometer. Protein was quantitated using a coomassie blue stain for total proteins (Bradford, 1976) and measured spectrophotometrically as above. Serum albumin was used as a standard for both protein measurement techniques. Amino acid analysis was performed on freeze-dried isolates. Samples were acid hydrolyzed with HCl or performic acid (Moore and Stein, 1954) and assayed on a Durum D500 amino acid analyzer.

Carbohydrate analysis

To determine the presence of carbohydrates, a molisch alpha napthol or anthrone sulfuric acid assay (Dische, 1955) was performed on isolates (40 mg/ml). An L-cysteine sulfuric acid assay (Dische, 1955) was also performed to determine the presence of hexoses, 6-deoxyhexoses, 2-deoxypentoses, pentoses, hexuronic acids, and heptoses. Standards used for this assay were: fucose, glucose, glucuronic acid, sedoheptulose, 2-deoxy-D-ribose, and ribose (Sigma). Either the Ehrlich reaction (Werner and Odin, 1952) or the thiobarbituric acid assay (Warren, 1959) was used to test for the presence of sialic acids. N-acetyl neuraminic acid (Sigma) was used as a standard (2 μg/ml for the thiobarbituric acid assay and 200 μg/ml for the Ehrlich reaction).

Enzymatic digestion and inhibition

Sensitivity to enzymatic degradation was tested on fresh and freeze-dried isolates at 24–26°C. Enzymes (Sigma) used were: 0.1% trypsin (bovine pancreatic) in 0.46 *M* Tris, pH 8.1, containing 0.012 *M* $CaCl_2$; 0.1% alpha chymotrypsin (bovine pancreatic) in 8 m*M* Tris, pH 7.8, containing 0.1 *M* $CaCl_2$; 0.2% aryl sulfatase (Aerobacter) in 0.2 *M* sodium acetate, pH 5.0; 0.02% hyaluronidase (bovine testis) in 0.1 *M* monosodium phosphate, pH 5.3, containing 0.15 *M* NaCl; 0.1% collagenase (Clostridium) in 0.05 *M* Tris, pH 7.5, containing 0.35 *M* $CaCl_2$; 0.1% lipase (Candida cylindracea) in 1.0 *M* Tris, pH 8.1. All solutions were prepared according to the Worthington manual (1972). Effects of these enzymes on precursor isolates were observed with light microscopy for periods up to 5 hours. As controls, precursor isolates were held for 5 hours in the buffer systems used for each enzyme.

Spawned eggs were collected in artificial seawater (Cavanaugh, 1956) containing either 0.1% soybean trypsin inhibitor (SBTI) (Sigma), 0.1% N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK) (Sigma), or 0.1% phenylmethylsulfonyl fluoride (PMSF) (Sigma). Eggs were either held in these solutions for observation or returned to normal seawater within 10 minutes after treatment in these solutions for observation. Control eggs were placed in normal seawater previously filtered through a millipore (0.2 μm) filter or held in a solution of artificial seawater (Cavanaugh, 1956).

Microscopic techniques

Ovarian tissue was dissected from either wild mature animals or animals induced to mature by bilateral eyestalk ablation (Duronslett *et al.*, 1975). These tissues were fixed in phosphate-buffered (pH 7.8) 10% formalin or Bouin's fixative (Thompson, 1966) and embedded in paraffin. Sections (5 μm) were stained with alcian blue 8GX at pH 2.0 (Thompson, 1966), periodic acid-Schiff reagent (PAS) (Thompson, 1966), aldehyde fuchsin (Thompson, 1966), or mucicarmine (Thompson, 1966).

Spawned eggs and isolated jelly precursor were fixed for 1–2 hours in a 0.2 *M* phosphate-buffered (pH 7.5) paraformaldehyde-glutaraldehyde solution (Karnovsky, 1965) for electron microscopy. Samples were post-fixed in 0.1 *M* phosphate-buffered (pH 7.5) osmium tetroxide (1%) for 30 minutes, rapidly dehydrated in a graded acetone series, and embedded in a low-viscosity epoxy resin (Spurr, 1969). Sections were cut with glass or diamond knives on a Porter Blum MT2-B ultramicrotome. Thin sections were stained with saturated methanolic uranyl acetate and aqueous lead citrate (Venable and Coggeshall, 1965) and examined on an Hitachi HS-8 electron microscope.

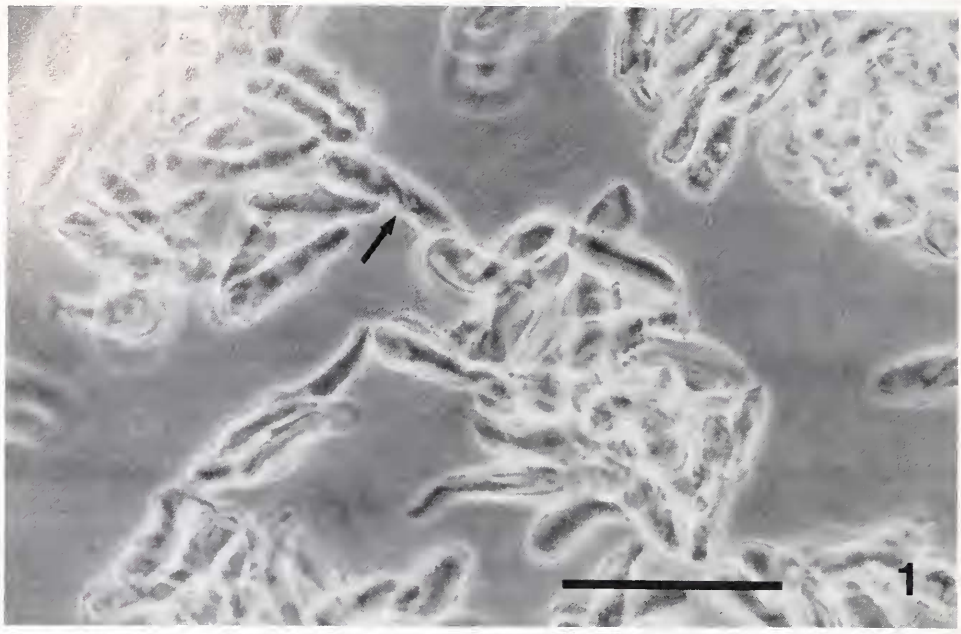
Results

Mature *P. aztecus* oocytes are approximately 265 μm in diameter and are isolecithal (Clark *et al.*, 1980). Extracellular club-shaped jelly precursors lie within membranous invaginations of the oolemma (crypts) and are separated from the environment by a thin vitelline envelope. The substructure of the jelly precursors consists of feathery elements. Contact with seawater initiates expulsion of the precursor from invaginations or crypts. As a result of the precursor expulsion, the vitelline envelope is lifted from the oolemma and is lost. Once released, the precursor elements dissipate forming a homogenous transparent jelly layer around the oocyte.

The jelly precursor elements within the crypts of an oocyte stain with PAS, but not alcian blue; however, the vitelline envelope exhibits a positive reaction with both stains. The vitelline envelope and the precursor material do not stain with either aldehyde fuchsin or mucicarmine.

Purified precursor isolates are shown in Figure 1 at the light level and in Figure 2 as observed with electron microscopy. The jelly precursor from each crypt maintains its structural integrity after isolation. Even the feathery substructural units of the precursor, originally described by Clark *et al.* (1980), are still apparent in fresh and freeze dried isolates (Fig. 3). Isolated precursor material was stable in several solvents (Table I). Sulfuric acid and sodium hydroxide completely dissipated the isolates and were compatible with the biochemical assays.

Table II shows assays for protein and carbohydrate components. The Lowry and Bradford assays were positive. From these determinations and the amino acid profiles, lyophilized precursor isolates are approximately 70–75% protein by weight. Both HCl-hydrolyzed and performic-acid-hydrolyzed isolates were analyzed for amino acid content since HCl hydrolysis partially or completely destroyed methio-



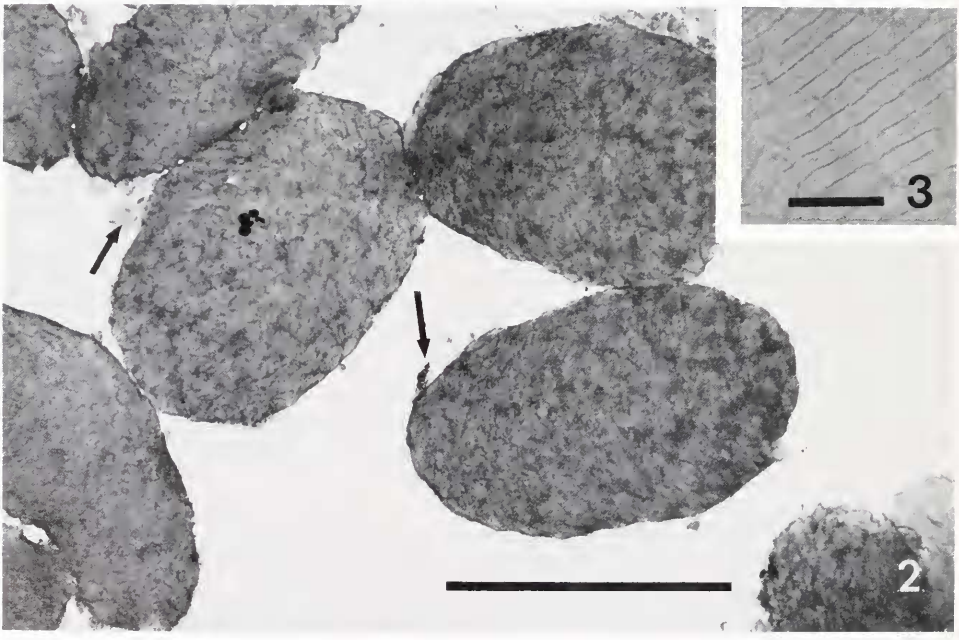


FIGURE 1. Light micrograph of a rehydrated sample of a purified jelly precursor isolate. Arrow: individual precursor element. Bar = 100 μm.

FIGURE 2. Transmission electron micrograph of rehydrated purified precursor isolates used for the biochemical assays. Only small amounts of contaminant were apparent and appeared to be primarily yolk in nature. CR: precursor element; arrow: suspected contaminant. Bar = 10μm.

FIGURE 3. High magnification transmission electron micrograph of feathery substructural elements of isolated jelly precursor. Bar = 0.5 μm.

TABLE I

Solvent extraction of jelly precursor isolates

Solvent	Precursor dissipation	Absorption peaks of solutes
8 *N* NaOH	Complete	260 nm, 225 nm
2 *N* NaOH	Complete	270 nm, 220 nm
18 *N* H_2SO_4	Complete	465 nm, 370 nm 320 nm, 250 nm
12 *N* HCl	Partial*	280 nm, 220 nm
2 *N* HCl	Partial*	280 nm, 220 nm
PO_4 buffer, pH 5.8	Partial*	265 nm, 250 nm
Acetone	None	None
Chloroform	None	None
Petroleum ether	None	None
Mercaptoethanol, pH 8.8	None	None
Methanol	None	None
Triton X-100 (1%)	None	None

* Swells and becomes flocculent.

nine and cysteine. Amino acid analysis showed a predominance of aspartic acid and glycine with relatively high ratios of cysteine (Table III).

The molish alpha napthol and the anthrone sulfuric acid assays indicated carbohydrates. Specific carbohydrate groups in the isolates were assayed using the L-cysteine sulfuric acid assay. With this assay, an initial peak in the sample was observed at 396 nm (Fig. 4), indicating a 6-deoxyhexose and possible overlapping absorption due to hexoses or pentoses. Following the addition of water and a 6-h waiting period, peaks were observed at 510 nm, 460 nm, and 410 nm. The peaks at 510 nm and 460 nm suggested heptoses. The peak at 410 nm probably resulted from a shift in the absorption of light in the range indicating a 6-deoxyhexose, since the addition of water destroys hexose absorption (Dische, 1955). Hexoses were suggested, however, by the decrease in the absorption at 396 nm after the addition of water. The sample probably did not contain pentose or 2-deoxyhexose, for no peaks comparable to the standards were observed. Sucrose contamination resulting from the isolation proce-

TABLE II

Summary of biochemical assays on jelly precursor isolates

Assay	Compounds reacting	Assay results
Lowry's	Protein	+
Bradford's	Protein	+
Molisch alpha-napthol assay	Carbohydrates	+
Anthrone sulfuric	Hexoses, pentoses, 6-deoxyhexoses,	+
acid assay	Hexuronic acid, heptoses	+
L-cysteine sulfuric	Pentoses	−
acid assay	Hexuronic acid	−
	Hexoses	+
	Heptoses	+
	6-Deoxyhexoses	+
	2-Deoxypentoses	+
Ehrlich reaction	Neuraminic acid	−
Thiobarbituric acid assay	Neuraminic acid	−

TABLE III

Amino acid composition of isolated jelly precursor

Amino acid	Composition, moles/100 mg
Aspartic acid	126.1
Threonine	57.4
Serine	50.6
Glutamic acid	61.9
Proline	28.7
Glycine	72.4
Alanine	51.0
Valine	57.1
Methionine	13.3
Cysteine	30.8
Isoleucine	30.0
Leucine	61.2
Tyrosine	20.7
Phenylalanine	29.4
Histidine	13.9
Lysine	37.3
Arginine	18.9

dure was discounted as the source of carbohydrates because the results remained the same for preparations isolated over glycerol. A glycerol standard gave no peaks with the L-cysteine reaction. Carbohydrate content was estimated to be approximately 25–30% of the lyophilized material weight, using the anthrone sulfuric acid assay. Assays for sialic acid were negative.

Table IV summarizes the results of enzyme treatments of isolated jelly precursor elements and shows that trypsin effected complete element dispersal. Light microscopy revealed a period of swelling followed by a loss of element morphology. After a 5-h treatment with chymotrypsin, the isolates swelled but did not dissipate completely. Jelly precursor elements held in the same buffers used with the enzyme assays maintained a normal morphology and did not swell or dissipate. In addition, jelly formation was inhibited in eggs spawned into seawater containing either SBTI, TLCK, or PMSF and the effect was not reversible on return to normal seawater. If 0.1% SBTI was added to seawater that contained eggs already in the process of jelly precursor element expulsion or dissipation, jelly formation was inhibited.

DISCUSSION

The oocytes of penaeid shrimp contain a jelly-like precursor in crypts of the oolemma prior to spawning (Clark *et al.*, 1980, 1984). This material is released at spawning and forms a jelly investment around the egg (Hudinaga, 1942; Clark *et al.*, 1980, 1984). In contrast to the jelly layers that invest the ova of many animals, the penaeid coat is a primary investment produced by the oocyte (unpub. data). The penaeid material also differs in its biochemical properties from the jellies of other animal ova. Thus, while the *P. aztecus* coat may be analogous to other egg jellies, it does not appear to be homologous. While there are several biochemical differences between the penaeid shrimp jelly and the jellies in other animal ova, it must be noted that the biochemical characteristics reported in this paper deal with a precursor form of the penaeid jelly. Subtle changes in the bonding and components may occur as the heterogeneous form of the jelly is transformed into the translucent homogenous form.

Jellies of spawned eggs from sea urchins and amphibians are composed of gly-

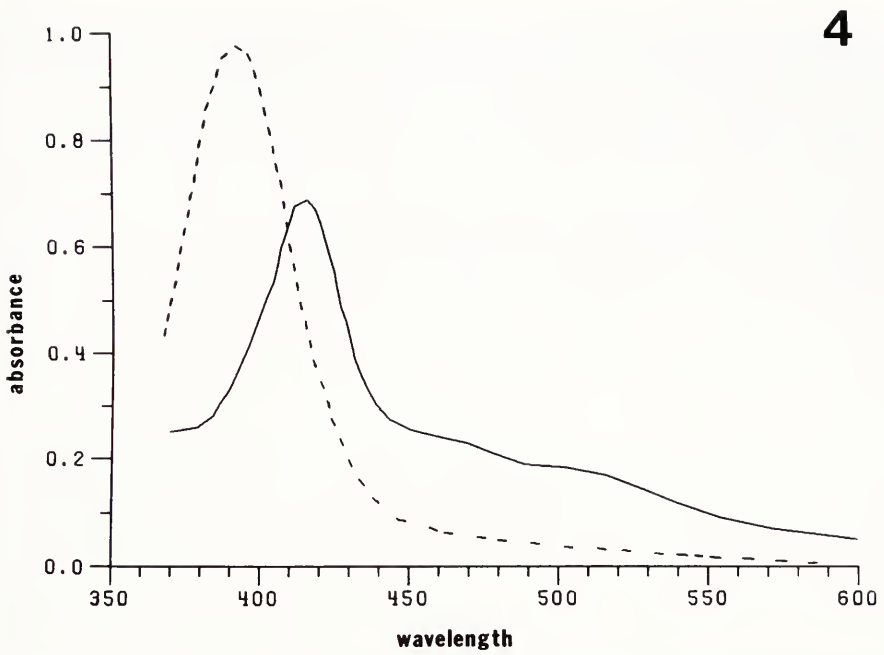


FIGURE 4. Spectrophotometric tracing of wavelength scan for the L-cysteine sulfuric acid reaction. Dotted line represents initial reaction; solid line represents reaction after a 6-hour waiting period and the addition of water. Tracing is a computer digitized reconstruction of the original scan.

cosaminoglycans, sulfate esters, and sialic acids (Vasseur, 1948; Monne and Slautterback, 1950; Humphries, 1966; Lee, 1967; Freeman, 1968; Isaka *et al.*, 1970; Hotta *et al.*, 1970a, b, 1973, 1977; Ishihara *et al.*, 1973; Katagiri, 1973; Lorenzi and Hedrick, 1973; SeGall and Lennarz, 1979). The jelly material released from teleost and nereid eggs is also reported to contain acid mucopolysaccharides with sulfate esters and, occasionally, a neutral mucopolysaccharide (Costello, 1949; Yamamoto, 1956; Raven, 1961; Yamamoto, 1961).

Biochemical assays suggest that, unlike most other egg jellies, the penaeid jelly precursor contains a substantial amount of protein compared to carbohydrate (70–

TABLE IV

Enzymic treatments of jelly precursor isolates

Enzyme	Concentration	Effect on isolated rods
Trypsin	0.1%	++
alpha-Chymotrypsin	0.1%	+
aryl-Sulfatase	0.2%	—
Hyaluronidase	0.02%	—
Collagenase	0.1%	—
Neuramindase	0.1%	—
Lipase	0.1%	—
alpha-Amylase	0.1%	—

++ dissipated; + swelling, no dissipation; — no effect.

75% protein, 25–30% carbohydrate). Alcian blue staining indicated carboxylated and/or sulfated carbohydrate groups in the vitelline envelope but not in the precursor material. Sialic acid was conspicuously absent from the isolates when tested by the method of Warren (1959), whereas it may compose over 25% of purified samples of sea urchin egg jelly (Hotta, 1977). In addition, enzymes capable of dispersing sea urchin egg jellies (aryl sulfatase and neuraminidase) had no detectable effect on the material released from *P. aztecus* eggs. The significance of these differences is unclear.

P. aztecus egg jelly also differs strikingly in protein content from sea urchin jelly. Sea urchin egg jelly contains approximately 20–25% protein (for review see Hunt, 1970) whereas *P. aztecus* egg jelly contains approximately 70–75% protein. Amino acid ratios, however, are similar in the two animals with the exception that the penaeid jelly material has higher cysteic acid ratios. Although sulfhydryl linkages are present in penaeid jelly material, they do not appear to be primarily responsible for structural integrity, since sulfhydryl reducing agents do not dissipate the jelly precursor.

Two lines of evidence indicate that trypsin-like protease enzymes are involved in the *in vivo* release and dispersion of the jelly precursor. Isolates were effectively dissipated by trypsin and to a much lesser degree by chymotrypsin. Secondly the specific serine-protease inhibitor SBTI inhibited this release and dispersion *in vivo*. Although TLCK and PMSF may also inhibit SH proteases in addition to serine proteases (Whitaker and Perez-Villaseñor, 1968), a serine protease is preferentially supported since SBTI is not reported to inhibit SH proteases and it is capable of completely inhibiting the jelly release. Regardless, a trypsin-like enzyme is suggested, but isolation and *in vitro* characterization of the enzyme will be essential in identifying the specific class of proteases represented by these enzymes.

Proteases are additionally implicated by the ability of the inhibitors to stop jelly formation at two stages: when the precursors are released from their crypts, and later, when the material is transformed into a translucent jelly layer. It is particularly interesting that these two stages of jelly release and transformation are also completely inhibited by a deficiency of Mg^{+2} in the seawater (Clark and Lynn, 1977) suggesting that the proteases involved are also Mg^{+2}-dependent. A similar protease-dependent jelly precursor release and transition of the jelly precursor into a homogenous jelly layer has also been observed in the eggs of *Sicyonia ingentis* (unpub. data). The initial phase of release which appears to be less Mg^{+2}-sensitive in the *S. ingentis* egg, seems to be mediated by failure of the vitelline envelope to break down, preventing jelly expulsion. A similar phenomenon may be responsible for preventing jelly release in the *P. aztecus* egg. The location of the proposed protease involved in the penaeid egg release is unknown. Therefore, specific activities of the enzymes involved must be localized and described to understand the sequence of events of the egg jelly release.

Despite the differences in chemical composition between egg jellies reported in other species and the jelly formed by *P. aztecus*, it is likely that the different types of jelly layers in question may have very similar functions. For example, the jelly formation in *P. aztecus* may protect the early zygote from the environment. Although morphologically the precursors are rapidly dissipated, their chemical constituents may remain around the ova for a long time. In this form, these chemical constituents could act as an antibacterial agent or as a repellent to other microorganisms. Preliminary studies reveal zones of inhibition around jelly precursor isolates placed on freshly streaked plates of shrimp exoskeletal bacteria (unpub. data). This exciting possibility should be further pursued *in vivo*.

An additional or alternative role for the formation of the jelly layer in *P. aztecus* may involve the acrosome reaction of the sperm. Acrosomal inducing abilities of egg investment coats have been demonstrated in sea urchins (SeGall and Lennarz, 1979)

and sturgeon (Cherr and Clark, 1985). Indeed, the investment coats of many species may contain components which trigger the acrosome reaction in sperm (Lopo, 1983, for review). Although the role of the *P. aztecus* jelly as an acrosomal reaction inducer has not been tested, a component of egg jelly from a related penaeid shrimp, *S. ingentis* has recently been demonstrated to induce the acrosome reaction (Clark *et al.*, 1984).

This study shows that the jelly formation in *P. aztecus* differs markedly from the cortical reactions of other animals, such as sea urchins. Instead, this reaction appears to be a delayed jelly-coat formation. It remains to be seen whether the physiological and biochemical differences of the shrimp jelly coat indicate, reflect, or match functional similarities of jelly investments of other animals.

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